USE OF BIOMARKERS FOR DETECTING ACUTE RENAL TRANSPLANT REJECTION

This application claims the benefit of U.S. provisional application no. 60/414,647, filed October 1, 2002; which application is incorporated by reference herein in its entirety.

FIELD OF THE INVENTION

The invention provides for Biomarkers important in the detection of acute renal transplant rejection. The Biomarkers were identified by distinguishing the urine protein profile of renal transplant patients with no rejection and those with acute rejection using SELDI analysis. The present invention relates the Biomarkers to a system and method in which the Biomarkers are used for the qualification of kidney transplant rejection status.

BACKGROUND OF THE INVENTION

Despite overall improvements associated with advances in immunosuppression regimens, rejection still occurs and has a deleterious effect on graft survival. The projected half-life of grafts transplanted in recent years was found to be almost double among those with no episodes of clinical acute rejection.(Hariharan S, Johnson CP, Bresnahan BA, et al. Improved graft survival after renal transplantation in the United States, 1988 to 1996. N Engl J Med. 2000; 342: 605-612; and Burke JF Jr, Pirsch JD, Ramos EL, et al. Long-term efficacy and safety of cyclosporine in renal-transplant recipients. N Engl J Med. 1994; 331: 358-363.) Acute rejection has been reported to cause a 20% reduction in the 1-year survival rate and a 4-year diminution in the projected half-life of cadaver allografts.(Li B, Hartono C, Ding R, et al. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. N Engl J Med. 2001; 344: 947-954. ; and Cecka JM. The UNOS Scientific Renal Transplant Registry. In: Cecka JM, Terasaki PI, eds. Clinical Transplantation 1999. Los Angeles: UCLA Immunogenetics

Center; 2000: 1-21.) During the first year after transplantation, approximately 35% of recipients will experience an episode of acute rejection. Reports also suggest that rejection is detected in 30% of biopsies done in patients thought to be having stable renal function or to have been successfully treated for rejection.(Rush DN, Henry SF, Jeffery JR, et al. Histological findings in early routine biopsies of stable renal allograft recipients. Transplantation. 1994; 57: 208-211.; and Gaber LW, Moore LW, Gaber AO, et al. Correlation of histology to clinical rejection reversal: a thymoglobulin multicenter trial report. Kidney Int. 1999; 55: 2415-2422.) The projected half-life of cadaveric renal transplants for recipients with and without an episode of clinical rejection was 7.0 versus 8.8 years and 8.8 versus 17.9 years in 1988 and 1995, respectively. When data corresponding to patients who died with a functioning graft were censored, there was a 31% increase in projected graft half-life during this period for recipients with an episode of rejection. The corresponding increase among patients with no rejection was 110%.(Hariharan S, Johnson CP, Bresnahan BA, et al. Improved graft survival after renal transplantation in the United States, 1988 to 1996. N Engl J Med. 2000; 342: 605-612.). The reduction in the relative hazard of graft failure during the first year after transplantation was 7.1% per year from 1988 to 1996.

Rejection can be defined as the immunologic interaction between host and allograft in which reactivity by the former leads to a sudden deterioration in physiologic function of the latter.(Almond PS, Matas A, Gillingham KJ, et al. Risk factors for chronic rejection in renal allograft recipients. Transplantation. 1993; 55: 752-756; Gulanikar AC, MacDonald AS, Sungurtekin U, et al. The incidence and impact of early rejection episodes on graft outcome in recipients of first cadaver kidney transplants. Transplantation. 1992; 53: 323-328; Lindholm A, Ohlman S, Albrechtsen D, et al. The impact of acute rejection episodes on long-term graft function and outcome in 1347 primary renal transplants treated by 3 cyclosporine regimens. Transplantation. 1993; 56: 307-315; and Cecka JM. The UNOS Scientific Renal Transplant Registry. In: Cecka JM, Terasaki PI, eds. Clinical Transplantation 1999. Los Angeles: UCLA Immunogenetics Center; 2000: 1-21.). Successful management requires early detection along with adequate treatment. Available diagnostic methods include clinical presentation, biochemical parameters, and tissue biopsies. The first two are not infallible. Serum creatinine, usually the first available

indication of allograft dysfunction, is not particularly sensitive or specific. Furthermore, it may not reflect early changes, since renal function may not always correlate with histologic improvement.(Roberti I, Reisman L. Serial evaluation of cell surface markers for immune activation after acute renal allograft rejection by urine flow cytometry-correlation with clinical outcome. Transplantation. 2001; 71: 1317-1320; Woodle ES, Cronin D, Newell KA, et al. Tacrolimus therapy for refractory acute renal allograft rejection. Transplantation. 1996; 62: 906; Beckingham IJ, Nicholson ML, Bell PR. Analysis of factors associated complications following renal transplant needle core biopsy. Br J Urol. 1994; 73: 13-15.). Biopsy of the renal allograft is regarded as the standard for the diagnosis of rejection and delayed graft function. However, percutaneous renal biopsy is costly and has associated morbidity and mortality. Complications include but are not limited to pain, hematuria, arteriovenous fistulas, perirenal hematomas, injury to adjacent viscera, anuria, allograft thrombosis, sepsis, shock, allograft loss, and patient death.(Beckingham IJ, Nicholson ML, Bell PR. Analysis of factors associated complications following renal transplant needle core biopsy. Br J Urol. 1994; 73: 13-15; Huraib S, Goldberg H, Katz A, et al. Percutaneous needle biopsy of the trans with planted kidney: technique and complications. Am J Kidney Dis. 1989; 14: 13-17; and Benfield MR, Herrin J, Feld L, et al. Safety of kidney biopsy in pediatric transplantation: a report of the Controlled Clinical Trials in Pediatric Transplantation Trial of Induction Therapy Study Group. Transplantation. 1999; 67: 544-547. Biopsies also allow for sampling errors and subsequent disparities between clinical and microscopic findings.(Curtis JJ, Julian BA, Sanders CE, et al. Dilemmas in renal transplantation: when the clinical course and histological findings differ. Am J Kidney Dis. 1996; 27: 435; and Sorof JM, Vartarian RK, Olson JL, et al. Histological concordance of paired renal allograft biopsy cores. Transplantation. 1995; 60: 1215.).

There is a critical need for the identification of Biomarkers that individually or in combination with other Biomarkers or diagnostic modalities deliver the required sensitivity and specificity for early detection of kidney transplant rejection. Thus, it is desirable to have a reliable and accurate method for early determination of kidney transplant rejection status in patients, the results of which can then be used to manage subject treatment. Development of a noninvasive Biomarker for renal transplant

rejection has the potential to radically change the way in which these transplant patients are managed.

SUMMARY OF THE INVENTION

The present invention provides sensitive and quick methods and kits that are useful for determining the kidney transplant rejection status by measuring Biomarkers of the present invention. The measurement of these Biomarkers in patient samples provides information that diagnosticians can correlate with a probable diagnosis of kidney transplant rejection or non-rejection. The Biomarkers are characterized by molecular weight and/or by other protein identities. The Biomarkers can be resolved from other proteins in a sample by using a variety of fractionation techniques, *e.g.*, chromatographic separation coupled with mass spectrometry, protein capture using immobilized antibodies or by traditional immunoassays. In preferred embodiments, the method of resolution involves Surface-Enhanced Laser Desorption/Ionization ("SELDI") mass spectrometry, in which the surface of the mass spectrometry probe comprises adsorbents that bind the Biomarkers.

More specifically, fourty-eight Biomarkers were discovered and subsequently identified, in accordance with the methods described and identified and referred to as Biomarkers 1 through 48.

The present invention provides a method of qualifying kidney transplant rejection status in a subject comprising (a) measuring at least one Biomarker in a sample from the subject, wherein the Biomarker is selected from the group consisting Biomarkers 1 through 48 and combinations thereof, and (b) correlating the measurement with kidney transplant rejection status. In certain methods, the measuring step comprises detecting the presence or absence of Biomarkers in the sample. In other methods, the measuring step comprises quantifying the amount of Biomarker(s) in the sample. In other methods, the measuring step comprises qualifying the type of bioBiomarker in the sample.

The invention also relates to methods wherein the measuring step comprises: providing a subject sample of urine or a urine derivative; fractionating proteins in the sample on an anion exchange resin and collecting fractions that contain Biomarkers 1

through 48; and capturing Biomarkers 1 through 48 from the fractions on a surface of a substrate comprising capture reagents that bind the protein Biomarkers. In preferred embodiments, the substrate is a SELDI probe comprising an IMAC copper surface and wherein the protein Biomarkers are detected by SELDI. In other embodiments, the substrate is a SELDI probe comprising biospecific affinity reagents that bind Biomarkers 1 through 48 and wherein the protein Biomarkers are detected by SELDI. In other embodiments, the substrate is a microtiter plate comprising biospecific affinity reagents that bind Biomarkers 1 through 48 and the protein Biomarkers are detected by immunoassay.

In certain embodiments, the methods further comprise managing subject treatment based on the status determined by the method. For example, if the result of the methods of the present invention is inconclusive or there is reason that confirmation of status is necessary, the physician may order more tests. Alternatively, if the status indicates that altering immunosuppression is appropriate, the physician may schedule the patient for a change in immunosuppressive therapy. Furthermore, if the results show that the current treatment is appropriatel, no further management may be necessary.

The invention also provides for such methods where the at least one Biomarker is measured again after subject management. In these instances, the step of managing subject treatment is then repeated and/or altered depending on the result obtained.

The term "kidney transplant rejection status" refers to the status of kidney function in the patient. Examples of types of kidney transplant rejection statuses include, but are not limited to, the subject's urine ceatinine levels, the degree of immunosuppression, and the effectiveness of immunosuppressive treatment. Other statuses and degrees of each status are known in the art.

The present invention also relates to Biomarkers designated as Biomarkers 1 through 48. Protein Biomarkers of the invention can be characterized in one or more of several respects. In particular, in one aspect, these Biomarkers are characterized by molecular weights under the conditions specified herein, particularly as determined by

mass spectral analysis. In another aspect, the Biomarkers can be characterized by features of the Biomarkers' mass spectral signature such as size (including area) and/or shape of the Biomarkers' spectral peaks, features including proximity, size and shape of neighboring peaks, etc. In yet another aspect, the Biomarkers can be characterized by affinity binding characteristics, particularly ability to binding to an IMAC copper adsorbent under specified conditions, however, other metals, e.g., nickel, may also be used. In preferred embodiments, Biomarkers of the invention may be characterized by each of such aspects, i.e. molecular weight, mass spectral signature and IMAC-Cu absorbent binding.

For the mass values of the Biomarkers disclosed herein, the mass accuracy of the spectral instrument is considered to be about within +/- 0.15 percent of the disclosed molecular weight value. Additionally, to such recognized accuracy variations of the instrument, the spectral mass determination can vary within resolution limits of from about 400 to 1000 m/dm, where m is mass and dm is the mass spectral peak width at 0.5 peak height. Those mass accuracy and resolution variances associated with the mass spectral instrument and operation thereof are reflected in the use of the term "about" in the disclosure of the mass of each of Biomarkers 1 through 48. It is also intended that such mass accuracy and resolution variances and thus meaning of the term "about" with respect to the mass of each of the Biomarkers disclosed herein is inclusive of variants of the Biomarkers as may exist due to sex, genotype and/or ethnicity of the subject and the particular cancer or origin or stage thereof.

The present invention further provides a method of qualifying kidney transplant rejection status in a subject comprising (a) measuring at least one bioBiomarker in a sample from the subject, wherein the bioBiomarker is selected from the group consisting of Biomarkers 1 through 48 and combinations thereof, and (b) correlating the measurement with kidney transplant rejection status. In certain methods, the measuring step comprises detecting the presence or absence of Biomarkers in the sample. In other methods, the measuring step comprises quantifying the amount of Biomarker(s) in the sample. In other methods, the measuring step comprises qualifying the type of bioBiomarker in the sample.

The accuracy of a diagnostic test is characterized by a Receiver Operating Characteristic curve ("ROC curve"). An ROC is a plot of the true positive rate against the false positive rate for the different possible cutpoints of a diagnostic test. An ROC curve shows the relationship between sensitivity and specificity. That is, an increase in sensitivity will be accompanied by a decrease in specificity. The closer the curve follows the left axis and then the top edge of the ROC space, the more accurate the test. Conversely, the closer the curve comes to the 45-degree diagonal of the ROC graph, the less accurate the test. The area under the ROC is a measure of test accuracy. The accuracy of the test depends on how well the test separates the group being tested into those with and without the disease in question. An area under the curve (referred to as "AUC") of 1 represents a perfect test, while an area of 0.5 represents a less useful test. Thus, preferred Biomarkers and diagnostic methods of the present invention have an AUC greater than 0.50, more preferred tests have an AUC greater than 0.60, more preferred tests have an AUC greater than 0.70.

Preferred methods of measuring the Biomarkers include use of a biochip array. Biochip arrays useful in the invention include protein and nucleic acid arrays. One or more Biomarkers are captured on the biochip array and subjected to laser ionization to detect the molecular weight of the Biomarkers. Analysis of the Biomarkers is, for example, by molecular weight of the one or more Biomarkers against a threshold intensity that is normalized against total ion current. Preferably, logarithmic transformation is used for reducing peak intensity ranges to limit the number of Biomarkers detected.

In preferred methods of the present invention, the step of correlating the measurement of the Biomarkers with kidney transplant status is performed by a software classification algorithm. Preferably, data is generated on immobilized subject samples on a biochip array, by subjecting said biochip array to laser ionization and detecting intensity of signal for mass/charge ratio; and, transforming the data into computer readable form; and executing an algorithm that classifies the data according to user input parameters, for detecting signals that represent Biomarkers present in kidney transplant rejection patients and are lacking in non-rejection patients.

Preferably the biochip surfaces are, for example, ionic, anionic, comprised of immobilized nickel ions, comprised of a mixture of positive and negative ions, comprised of one or more antibodies, single or double stranded nucleic acids, proteins, peptides or fragments thereof, amino acid probes, or phage display libraries.

In other preferred methods one or more of the Biomarkers are measured using laser desorption/ionization mass spectrometry, comprising providing a probe adapted for use with a mass spectrometer comprising an adsorbent attached thereto, and contacting the subject sample with the adsorbent, and; desorbing and ionizing the Biomarker or Biomarkers from the probe and detecting the deionized/ionized Biomarkers with the mass spectrometer.

Preferably, the laser desorption/ionization mass spectrometry comprises: providing a substrate comprising an adsorbent attached thereto; contacting the subject sample with the adsorbent; placing the substrate on a probe adapted for use with a mass spectrometer comprising an adsorbent attached thereto; and, desorbing and ionizing the Biomarker or Biomarkers from the probe and detecting the desorbed/ionized Biomarker or Biomarkers with the mass spectrometer.

The adsorbent can for example be hydrophobic, hydrophilic, ionic or metal chelate adsorbent, such as, nickel or an antibody, single- or double stranded oligonucleotide, amino acid, protein, peptide or fragments thereof.

The methods of the present invention can be performed on any type of patient sample that would be amenable to such methods, e.g., blood, serum and plasma. The preferred patient sample is urine.

In certain embodiments, a plurality of Biomarkers in a sample from the subject are measured, wherein the Biomarkers are selected from the group consisting of Biomarkers 1 through 48. In preferred methods, the plurality of Biomarkers consists of Biomarkers 3, 6, 14, 15, 16, 18, 19, 20, 21, 22, 23, 32 and 35 Preferably, the protein Biomarkers are measured by SELDI or immunoassay.

The present invention also provides kits comprising (a) a capture reagent that binds a Biomarker selected from Biomarkers 1 through 48, and combinations thereof; and (b) a container comprising at least one of the Biomarkers. In preferred embodiments, the capture reagent binds a plurality of the Biomarkers. In one embodiment, the plurality comprises Biomarkers 3, 6, 14, 15, 16, 18, 19, 20, 21, 22, 23, 32 and 35. While the capture reagent can be any type of reagent, preferably the reagent is a SELDI probe. The capture reagent may also bind other known Biomarkers. In certain preferred embodiments, the kit of further comprises a second capture reagent that binds one of the Biomarkers that the first capture reagent does not bind.

Further kits provided by the invention comprise (a) a first capture reagent that binds at least one Biomarker selected from Biomarkers 1 through 48, and (b) a second capture reagent that binds at least one of the Biomarkers that is not bound by the first capture reagent. Preferably, at least one the capture reagent is an antibody. Certain kits further comprise an MS probe to which at least one capture reagent is attached or is attachable.

In certain kits of the present invention, the capture reagent comprises an immobilized metal chelate ("IMAC").

Certain kits of the present invention further comprise a wash solution that selectively allows retention of the bound Biomarker to the capture reagent as compared with other Biomarkers after washing.

The invention also provides kits comprising (a) a first capture reagent that binds at least one Biomarker selected from Biomarkers 1 through 48, and (b) instructions for using the capture reagent to measure the Biomarker. In certain of these kits, the capture reagent comprises an antibody. Furthermore, some kits further comprise an MS probe to which the capture reagent is attached or is attachable. In some kits, the capture reagent comprises an IMAC. The kits may also contain a wash solution that selectively allows retention of the bound Biomarker to the capture reagent as compared with other Biomarkers after washing. Preferably, the kit comprises written instructions for use of the kit for determining kidney transplant

rejection status and the instructions provide for contacting a test sample with the capture reagent and measuring one or more Biomarkers retained by the capture reagent.

The kit also provides for a capture reagent, which is an antibody, single or double stranded oligonucleotide, amino acid, protein, peptide or fragments thereof.

Measurement of one or more protein Biomarkers using the kit, is by mass spectrometry or immunoassays such as an ELISA.

Purified proteins for detection of kidney transplant rejection and/or generation of antibodies for further diagnostic assays are also provided for

Other aspects of the invention are described *infra*.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 1 having a molecular weight of about 2.5.

Figure 2 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 2 having a molecular weight of about 2.6.

Figure 3 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 3 having a molecular weight of about 3.4.

Figure 4 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 4 having a molecular weight of about 3.5.

Figure 5 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 5 having a molecular weight of about 3.8.

Figure 6 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 6 having a molecular weight of about 4.1.

Figure 7 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 7 having a molecular weight of about 4.7.

Figure 8 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 8 having a molecular weight of about 4.8.

Figure 9 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 9 having a molecular weight of about 5.0.

Figure 10 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 10 having a molecular weight of about 5.5.

Figure 11 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 11 having a molecular weight of about 5.6.

Figure 12 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 12 having a molecular weight of about 6.1.

Figure 13 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 13 having a molecular weight of about 6.4.

Figure 14 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 14 having a molecular weight of about 6.5.

Figure 15 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 15 having a molecular weight of about 6.6.

Figure 16 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 16 having a molecular weight of about 6.7.

Figure 17 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 17 having a molecular weight of about 6.8.

Figure 18 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 18 having a molecular weight of about 7.0.

Figure 19 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 19 having a molecular weight of about 7.1.

Figure 20 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 20 having a molecular weight of about 7.3.

Figure 21 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 21 having a molecular weight of about 7.5.

Figure 22 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 22 having a molecular weight of about 7.8.

Figure 23 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 23 having a molecular weight of about 8.0.

Figure 24 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 24 having a molecular weight of about 8.1.

Figure 25 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 25 having a molecular weight of about 9.0.

Figure 26 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 26 having a molecular weight of about 9.1.

Figure 27 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 27 having a molecular weight of about 9.3.

Figure 28 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 28 having a molecular weight of about 9.6.

Figure 29 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 29 having a molecular weight of about 9.7.

Figure 30 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 30 having a molecular weight of about 9.8.

Figure 31 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 31 having a molecular weight of about 10.0.

Figure 32 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 32 having a molecular weight of about 10.8.

Figure 33 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 33 having a molecular weight of about 10.9.

Figure 34 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 34 having a molecular weight of about 11.3.

Figure 35 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 35 having a molecular weight of about 13.4.

Figure 36 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 36 having a molecular weight of about 13.9.

Figure 37 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 37 having a molecular weight of about 14.7.

Figure 38 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 38 having a molecular weight of about 14.8.

Figure 39 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 39 having a molecular weight of about 15.1.

Figure 40 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 40 having a molecular weight of about 15.2.

Figure 41 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 41 having a molecular weight of about 16.1.

Figure 42 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 42 having a molecular weight of about 25.0.

Figure 43 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 43 having a molecular weight of about 28.0.

Figure 44 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 44 having a molecular weight of about 50.0.

Figure 45 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 45 having a molecular weight of about 50.1.

Figure 46 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 46 having a molecular weight of about 51.1.

Figure 47 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 47 having a molecular weight of about 51.3.

Figure 48 A and B represent the Receiver Operator Characteristic (ROC) Curves and data for BioBiomarker 48 having a molecular weight of about 67.0.

Figure 49 shows a sample mass spectra from nonrejection patients and rejection patients.

Figure 50 shows another sample mass spectra from a nonrejection patient and a rejection patient.

Figure 51 shows an illustrative ROC analysis of candidate biomarkers.

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.*, *Dictionary of Microbiology and Molecular Biology* (2nd ed. 1994); *The Cambridge Dictionary of Science and Technology* (Walker ed., 1988); *The Glossary of Genetics*, 5th Ed., R. Rieger *et al.* (eds.), Springer Verlag (1991); and Hale & Marham, *The Harper Collins Dictionary of Biology* (1991). As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

"Gas phase ion spectrometer" refers to an apparatus that detects gas phase ions. Gas phase ion spectrometers include an ion source that supplies gas phase ions. Gas phase ion spectrometers include, for example, mass spectrometers, ion mobility spectrometers, and total ion current measuring devices. "Gas phase ion spectrometry" refers to the use of a gas phase ion spectrometer to detect gas phase ions.

"Mass spectrometer" refers to a gas phase ion spectrometer that measures a parameter that can be translated into mass-to-charge ratios of gas phase ions. Mass spectrometers generally include an ion source and a mass analyzer. Examples of mass spectrometers are time-of-flight, magnetic sector, quadrupole filter, ion trap, ion cyclotron resonance, electrostatic sector analyzer and hybrids of these. "Mass spectrometry" refers to the use of a mass spectrometer to detect gas phase ions.

"Laser desorption mass spectrometer" refers to a mass spectrometer that uses laser energy as a means to desorb, volatilize, and ionize an analyte.

"Tandem mass spectrometer" refers to any mass spectrometer that is capable of performing two successive stages of m/z-based discrimination or measurement of ions, including ions in an ion mixture. The phrase includes mass spectrometers having two mass analyzers that are capable of performing two successive stages of m/z-based discrimination or measurement of ions tandem-in-space. The phrase further includes mass spectrometers having a single mass analyzer that is capable of performing two successive stages of m/z-based discrimination or measurement of ions

tandem-in-time. The phrase thus explicitly includes Qq-TOF mass spectrometers, ion trap mass spectrometers, ion trap-TOF mass spectrometers, TOF-TOF mass spectrometers, Fourier transform ion cyclotron resonance mass spectrometers, electrostatic sector – magnetic sector mass spectrometers, and combinations thereof.

"Mass analyzer" refers to a sub-assembly of a mass spectrometer that comprises means for measuring a parameter that can be translated into mass-to-charge ratios of gas phase ions. In a time-of-flight mass spectrometer the mass analyzer comprises an ion optic assembly, a flight tube and an ion detector.

"Ion source" refers to a sub-assembly of a gas phase ion spectrometer that provides gas phase ions. In one embodiment, the ion source provides ions through a desorption/ionization process. Such embodiments generally comprise a probe interface that positionally engages a probe in an interrogatable relationship to a source of ionizing energy (e.g., a laser desorption/ionization source) and in concurrent communication at atmospheric or subatmospheric pressure with a detector of a gas phase ion spectrometer.

Forms of ionizing energy for desorbing/ionizing an analyte from a solid phase include, for example: (1) laser energy; (2) fast atoms (used in fast atom bombardment); (3) high energy particles generated via beta decay of radionucleides (used in plasma desorption); and (4) primary ions generating secondary ions (used in secondary ion mass spectrometry). The preferred form of ionizing energy for solid phase analytes is a laser (used in laser desorption/ionization), in particular, nitrogen lasers, Nd-Yag lasers and other pulsed laser sources. "Fluence" refers to the energy delivered per unit area of interrogated image. A high fluence source, such as a laser, will deliver about 1 mJ / mm2 to 50 mJ / mm2. Typically, a sample is placed on the surface of a probe, the probe is engaged with the probe interface and the probe surface is struck with the ionizing energy. The energy desorbs analyte molecules from the surface into the gas phase and ionizes them.

Other forms of ionizing energy for analytes include, for example: (1) electrons that ionize gas phase neutrals; (2) strong electric field to induce ionization from gas phase, solid phase, or liquid phase neutrals; and (3) a source that applies a

combination of ionization particles or electric fields with neutral chemicals to induce chemical ionization of solid phase, gas phase, and liquid phase neutrals.

"Solid support" refers to a solid material which can be derivatized with, or otherwise attached to, a capture reagent. Exemplary solid supports include probes, microtiter plates and chromatographic resins.

"Probe" in the context of this invention refers to a device adapted to engage a probe interface of a gas phase ion spectrometer (e.g., a mass spectrometer) and to present an analyte to ionizing energy for ionization and introduction into a gas phase ion spectrometer, such as a mass spectrometer. A "probe" will generally comprise a solid substrate (either flexible or rigid) comprising a sample presenting surface on which an analyte is presented to the source of ionizing energy.

"Surface-enhanced laser desorption/ionization" or "SELDI" refers to a method of desorption/ionization gas phase ion spectrometry (e.g., mass spectrometry) in which the analyte is captured on the surface of a SELDI probe that engages the probe interface of the gas phase ion spectrometer. In "SELDI MS," the gas phase ion spectrometer is a mass spectrometer. SELDI technology is described in, e.g., U.S. patent 5,719,060 (Hutchens and Yip) and U.S. patent 6,225,047 (Hutchens and Yip).

"Surface-Enhanced Affinity Capture" or "SEAC" is a version of SELDI that involves the use of probes comprising an absorbent surface (a "SEAC probe").

"Adsorbent surface" refers to a surface to which is bound an adsorbent (also called a "capture reagent" or an "affinity reagent"). An adsorbent is any material capable of binding an analyte (e.g., a target polypeptide or nucleic acid). "Chromatographic adsorbent" refers to a material typically used in chromatography. Chromatographic adsorbents include, for example, ion exchange materials, metal chelators (e.g., nitriloacetic acid or iminodiacetic acid), immobilized metal chelates, hydrophobic interaction adsorbents, hydrophilic interaction adsorbents, dyes, simple biomolecules (e.g., nucleotides, amino acids, simple sugars and fatty acids) and mixed mode adsorbents (e.g., hydrophobic attraction/electrostatic repulsion adsorbents). "Biospecific adsorbent" refers an adsorbent comprising a biomolecule, e.g., a nucleic acid molecule (e.g., an aptamer), a polypeptide, a polysaccharide, a lipid, a steroid or

a conjugate of these (e.g., a glycoprotein, a lipoprotein, a glycolipid, a nucleic acid (e.g., DNA)-protein conjugate). In certain instances the biospecific adsorbent can be a macromolecular structure such as a multiprotein complex, a biological membrane or a virus. Examples of biospecific adsorbents are antibodies, receptor proteins and nucleic acids. Biospecific adsorbents typically have higher specificity for a target analyte than chromatographic adsorbents. Further examples of adsorbents for use in SELDI can be found in U.S. Patent 6,225,047 (Hutchens and Yip, "Use of retentate chromatography to generate difference maps," May 1, 2001).

In some embodiments, a SEAC probe is provided as a pre-activated surface which can be modified to provide an adsorbent of choice. For example, certain probes are provided with a reactive moiety that is capable of binding a biological molecule through a covalent bond. Epoxide and carbodiimidizole are useful reactive moieties to covalently bind biospecific adsorbents such as antibodies or cellular receptors.

"Adsorption" refers to detectable non-covalent binding of an analyte to an adsorbent or capture reagent.

"Surface-Enhanced Neat Desorption" or "SEND" is a version of SELDI that involves the use of probes comprising energy absorbing molecules chemically bound to the probe surface. ("SEND probe.") "Energy absorbing molecules" ("EAM") refer to molecules that are capable of absorbing energy from a laser desorption/ ionization source and thereafter contributing to desorption and ionization of analyte molecules in contact therewith. The phrase includes molecules used in MALDI , frequently referred to as "matrix", and explicitly includes cinnamic acid derivatives, sinapinic acid ("SPA"), cyano-hydroxy-cinnamic acid ("CHCA") and dihydroxybenzoic acid, ferulic acid, hydroxyacetophenone derivatives, as well as others. It also includes EAMs used in SELDI. SEND is further described in United States patent 5,719,060 and United States patent application 60/408,255, filed September 4, 2002 (Kitagawa, "Monomers And Polymers Having Energy Absorbing Moieties Of Use In Desorption/Ionization Of Analytes").

"Surface-Enhanced Photolabile Attachment and Release" or "SEPAR" is a version of SELDI that involves the use of probes having moieties attached to the surface that can covalently bind an analyte, and then release the analyte through breaking a photolabile bond in the moiety after exposure to light, e.g., laser light. SEPAR is further described in United States patent 5,719,060.

"Eluant" or "wash solution" refers to an agent, typically a solution, which is used to affect or modify adsorption of an analyte to an adsorbent surface and/or remove unbound materials from the surface. The elution characteristics of an eluant can depend, for example, on pH, ionic strength, hydrophobicity, degree of chaotropism, detergent strength and temperature.

"Analyte" refers to any component of a sample that is desired to be detected. The term can refer to a single component or a plurality of components in the sample.

The "complexity" of a sample adsorbed to an adsorption surface of an affinity capture probe means the number of different protein species that are adsorbed.

"Molecular binding partners" and "specific binding partners" refer to pairs of molecules, typically pairs of biomolecules that exhibit specific binding. Molecular binding partners include, without limitation, receptor and ligand, antibody and antigen, biotin and avidin, and biotin and streptavidin.

"Monitoring" refers to recording changes in a continuously varying parameter.

"Biochip" refers to a solid substrate having a generally planar surface to which an adsorbent is attached. Frequently, the surface of the biochip comprises a plurality of addressable locations, each of which location has the adsorbent bound there. Biochips can be adapted to engage a probe interface and, therefore, function as probes.

"Protein biochip" refers to a biochip adapted for the capture of polypeptides. Many protein biochips are described in the art. These include, for example, protein biochips produced by Ciphergen Biosystems (Fremont, CA), Packard BioScience

Company (Meriden CT), Zyomyx (Hayward, CA) and Phylos (Lexington, MA). Examples of such protein biochips are described in the following patents or patent applications: U.S. patent 6,225,047 (Hutchens and Yip, "Use of retentate chromatography to generate difference maps," May 1, 2001); International publication WO 99/51773 (Kuimelis and Wagner, "Addressable protein arrays," October 14, 1999); U.S. patent 6,329,209 (Wagner et al., "Arrays of protein-capture agents and methods of use thereof," December 11, 2001) and International publication WO 00/56934 (Englert et al., "Continuous porous matrix arrays," September 28, 2000).

Protein biochips produced by Ciphergen Biosystems comprise surfaces having chromatographic or biospecific adsorbents attached thereto at addressable locations. Ciphergen ProteinChip® arrays include NP20, H4, H50, SAX-2, WCX-2, CM-10, IMAC-3, IMAC-30, LSAX-30, LWCX-30, IMAC-40, PS-10, PS-20 and PG-20. These protein biochips comprise an aluminum substrate in the form of a strip. The surface of the strip is coated with silicon dioxide.

In the case of the NP-20 biochip, silicon oxide functions as a hydrophilic adsorbent to capture hydrophilic proteins.

H4, H50, SAX-2, WCX-2, CM-10, IMAC-3, IMAC-30, PS-10 and PS-20 biochips further comprise a functionalized, cross-linked polymer in the form of a hydrogel physically attached to the surface of the biochip or covalently attached through a silane to the surface of the biochip. The H4 biochip has isopropyl functionalities for hydrophobic binding. The H50 biochip has nonylphenoxy-poly(ethylene glycol)methacrylate for hydrophobic binding. The SAX-2 biochip has quaternary ammonium functionalities for anion exchange. The WCX-2 and CM-10 biochips have carboxylate functionalities for cation exchange. The IMAC-3 and IMAC-30 biochips have nitriloacetic acid functionalities that adsorb transition metal ions, such as Cu++ and Ni++, by chelation. These immobilized metal ions allow adsorption of peptide and proteins by coordinate bonding. The PS-10 biochip has carboimidizole functional groups that can react with groups on proteins for covalent binding. The PS-20 biochip has epoxide functional groups for covalent binding with proteins. The PS-series biochips are useful for binding biospecific adsorbents, such as

antibodies, receptors, lectins, heparin, Protein A, biotin/streptavidin and the like, to chip surfaces where they function to specifically capture analytes from a sample. The PG-20 biochip is a PS-20 chip to which Protein G is attached. The LSAX-30 (anion exchange), LWCX-30 (cation exchange) and IMAC-40 (metal chelate) biochips have functionalized latex beads on their surfaces. Such biochips are further described in: WO 00/66265 (Rich et al., "Probes for a Gas Phase Ion Spectrometer," November 9, 2000); WO 00/67293 (Beecher et al., "Sample Holder with Hydrophobic Coating for Gas Phase Mass Spectrometer," November 9, 2000); U.S. patent application US20030032043A1 (Pohl and Papanu, "Latex Based Adsorbent Chip," July 16, 2002) and U.S. patent application 60/350,110 (Um et al., "Hydrophobic Surface Chip," November 8, 2001).

Upon capture on a biochip, analytes can be detected by a variety of detection methods selected from, for example, a gas phase ion spectrometry method, an optical method, an electrochemical method, atomic force microscopy and a radio frequency method. Gas phase ion spectrometry methods are described herein. Of particular interest is the use of mass spectrometry and, in particular, SELDI. Optical methods include, for example, detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, transmittance, birefringence or refractive index (e.g., surface plasmon resonance, ellipsometry, a resonant mirror method, a grating coupler waveguide method or interferometry). Optical methods include microscopy (both confocal and non-confocal), imaging methods and non-imaging methods. Immunoassays in various formats (e.g., ELISA) are popular methods for detection of analytes captured on a solid phase. Electrochemical methods include voltametry and amperometry methods. Radio frequency methods include multipolar resonance spectroscopy.

"Biomarker" in the context of the present invention refers to a polypeptide (of a particular apparent molecular weight), which is differentially present in a sample taken from patients having received a kidney transplant under rejection as compared to a patient having received a kidney transplant not under rejection.

The term "measuring" means methods which include detecting the presence or absence of Biomarker(s) in the sample, quantifying the amount of Biomarker(s) in

the sample, and/or qualifying the type of bioBiomarker. Measuring can be accomplished by methods known in the art and those further described herein, including but not limited to SELDI and immunoassay. Any suitable methods can be used to detect and measure one or more of the Biomarkers described herein. These methods include, without limitation, mass spectrometry (*e.g.*, laser desorption/ionization mass spectrometry), fluorescence (*e.g.* sandwich immunoassay), surface plasmon resonance, ellipsometry and atomic force microscopy.

The phrase "differentially present" refers to differences in the quantity and/or the frequency of a Biomarker present in a sample taken from patients having received a kidney transplant. For example, the Biomarker 6 is present at an elevated level in samples of kidney transplant rejection patients compared to samples from kidney transplant non-rejection patients. In contrast, Biomarkers 25, 29 and 30 described herein are present at a decreased level in samples of kidney transplant rejection patients compared to samples from kidney transplant non-rejection patients. Furthermore, a Biomarker can be a polypeptide, which is detected at a higher frequency or at a lower frequency in samples of kidney transplant rejection patients compared to samples from kidney transplant non-rejection patients. A Biomarker can be differentially present in terms of quantity, frequency or both.

A polypeptide is differentially present between two samples if the amount of the polypeptide in one sample is statistically significantly different from the amount of the polypeptide in the other sample. For example, a polypeptide is differentially present between the two samples if it is present at least about 120%, at least about 130%, at least about 150%, at least about 180%, at least about 200%, at least about 300%, at least about 500%, at least about 700%, at least about 900%, or at least about 1000% greater than it is present in the other sample, or if it is detectable in one sample and not detectable in the other.

Alternatively or additionally, a polypeptide is differentially present between two sets of samples if the frequency of detecting the polypeptide in the kidney transplant rejection patients' samples is statistically significantly higher or lower than in the samples from non-rejection patients. For example, a polypeptide is differentially present between the two sets of samples if it is detected at least about

120%, at least about 130%, at least about 150%, at least about 180%, at least about 200%, at least about 300%, at least about 500%, at least about 700%, at least about 900%, or at least about 1000% more frequently or less frequently observed in one set of samples than the other set of samples.

"Diagnostic" means identifying the presence or nature of a pathologic condition, i.e., kidney transplant rejection. Diagnostic methods differ in their sensitivity and specificity. The "sensitivity" of a diagnostic assay is the percentage of diseased individuals who test positive (percent of "true positives"). Diseased individuals not detected by the assay are "false negatives." Subjects who are not diseased and who test negative in the assay, are termed "true negatives." The "specificity" of a diagnostic assay is 1 minus the false positive rate, where the "false positive" rate is defined as the proportion of those without the disease who test positive. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

A "test amount" of a Biomarker refers to an amount of a Biomarker present in a sample being tested. A test amount can be either in absolute amount (*e.g.*, μg/ml) or a relative amount (*e.g.*, relative intensity of signals).

A "diagnostic amount" of a Biomarker refers to an amount of a Biomarker in a subject's sample that is consistent with a diagnosis of kidney transplant rejection. A diagnostic amount can be either in absolute amount (*e.g.*, μg/ml) or a relative amount (*e.g.*, relative intensity of signals).

A "control amount" of a Biomarker can be any amount or a range of amount, which is to be compared against a test amount of a Biomarker. For example, a control amount of a Biomarker can be the amount of a Biomarker in a person without kidney transplant rejection. A control amount can be either in absolute amount (*e.g.*, μg/ml) or a relative amount (*e.g.*, relative intensity of signals).

"Antibody" refers to a polypeptide ligand substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, which specifically binds and recognizes an epitope (*e.g.*, an antigen). The recognized immunoglobulin genes include the kappa and lambda light chain constant region genes, the alpha, gamma, delta, epsilon and mu heavy chain constant region genes, and the myriad immunoglobulin variable region genes. Antibodies exist, *e.g.*, as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases. This includes, *e.g.*, Fab' and $F(ab)'_2$ fragments. The term "antibody," as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized de novo using recombinant DNA methodologies. It also includes polyclonal antibodies, monoclonal antibodies, chimeric antibodies, humanized antibodies, or single chain antibodies. "Fc" portion of an antibody refers to that portion of an immunoglobulin heavy chain that comprises one or more heavy chain constant region domains, CH_1, CH_2 and CH_3, but does not include the heavy chain variable region.

"Managing subject treatment" refers to the behavior of the clinician or physician subsequent to the determination of kidney transplant rejection status. For example, if the result of the methods of the present invention is inconclusive or there is reason that confirmation of status is necessary, the physician may order more tests. Alternatively, if the status indicates that altering immunosuppressive therapy is appropriate, the physician may schedule the patient for that change in treatment. Likewise, if the status is negative, e.g., late stage kidney transplant rejection or if the status is acute, no further action may be warranted. Furthermore, if the results show that treatment has been successful, no further management may be necessary.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides Biomarkers generated from comparison of protein profiles from patients diagnosed with kidney transplant rejection and from patients without kidney transplant rejection, using the ProteinChip® Biomarker System (Ciphergen Biosystems, Inc., Fremont, CA). These Biomarkers, together with other known kidney transplant rejection Biomarkers, were evaluated individually and in multivariate predictive models. In particular, it is shown that these Biomarkers, used individually, or preferably in combination with other Biomarkers from this group

or with other diagnostic tests, provide a novel method of determining kidney transplant rejection status in a subject.

High-throughput protein profiling combined with effective use of bioinformatics tools provides a useful approach to screening for kidnet transplant rejection Biomarkers. Briefly, the system used in the present invention utilizes chromatographic ProteinChip® Arrays to assay samples using SELDI (Surface Enhanced Laser Desorption/Ionization). Proteins bound to the arrays are read in a ProteinChip® Reader, a time-of-flight mass spectrometer.

The present invention is based upon the discovery of protein Biomarkers that are differentially present in samples of kidney transplant rejection patients and kidney transplant non-rejection patients, and the application of this discovery in methods and kits for determining kidney transplant rejection status. These protein Biomarkers are found in samples from kidney transplant rejection patients at levels that are different than the levels in samples from patients without kidney transplant rejection. Accordingly, the amount of one or more Biomarkers found in a test sample compared to a control, or the presence or absence of one or more Biomarkers in the test sample provides useful information regarding the kidney transplant rejection status of the patient.

DISCOVERED RENAL TRANSPLANT REJECTION BIOMARKERS

The corresponding proteins or fragments of proteins for these bioBiomarkers are represented as intensity peaks in SELDI (surface enhanced laser desorption/ionization) protein chip/mass spectra with molecular masses centered around the following values:

Biomarker 1: having a molecular weight of about 2.5 kD;
Biomarker 2: having a molecular weight of about 2.6 kD;
Biomarker 3: having a molecular weight of about 3.4 kD;
Biomarker 4: having a molecular weight of about 3.5 kD;
Biomarker 5: having a molecular weight of about 3.8 kD;
Biomarker 6: having a molecular weight of about 4.1 kD;
Biomarker 7: having a molecular weight of about 4.7 kD;

Biomarker 8: having a molecular weight of about 4.8 kD;
Biomarker 9: having a molecular weight of about 5.0 kD;
Biomarker 10: having a molecular weight of about 5.5 kD;
Biomarker 11: having a molecular weight of about 5.6 kD;
Biomarker 12: having a molecular weight of about 6.1 kD;
Biomarker 13: having a molecular weight of about 6.4 kD;
Biomarker 14: having a molecular weight of about 6.5 kD;
Biomarker 15: having a molecular weight of about 6.6 kD;
Biomarker 16: having a molecular weight of about 6.7 kD;
Biomarker 17: having a molecular weight of about 6.8 kD;
Biomarker 18: having a molecular weight of about 7.0 kD;
Biomarker 19: having a molecular weight of about 7.1 kD;
Biomarker 20: having a molecular weight of about 7.3 kD;
Biomarker 21: having a molecular weight of about 7.5 kD;
Biomarker 22: having a molecular weight of about 7.8 kD;
Biomarker 23: having a molecular weight of about 8.0 kD;
Biomarker 24: having a molecular weight of about 8.1 kD;
Biomarker 25: having a molecular weight of about 9.0 kD;
Biomarker 26: having a molecular weight of about 9.1 kD;
Biomarker 27: having a molecular weight of about 9.3 kD;
Biomarker 28: having a molecular weight of about 9.6 kD;
Biomarker 29: having a molecular weight of about 9.7 kD;
Biomarker 30: having a molecular weight of about 9.8 kD;
Biomarker 31: having a molecular weight of about 10.0 kD;
Biomarker 32: having a molecular weight of about 10.8 kD;
Biomarker 33: having a molecular weight of about 10.9 kD;
Biomarker 34: having a molecular weight of about 11.3 kD;
Biomarker 35: having a molecular weight of about 13.4 kD;
Biomarker 36: having a molecular weight of about 13.9 kD;
Biomarker 37: having a molecular weight of about 14.7 kD;
Biomarker 38: having a molecular weight of about 14.8 kD;
Biomarker 39: having a molecular weight of about 15.1 kD;
Biomarker 40: having a molecular weight of about 15.2 kD;
Biomarker 41: having a molecular weight of about 16.1 kD;

Biomarker 42: having a molecular weight of about 25.0 kD;
Biomarker 43: having a molecular weight of about 28.0 kD;
Biomarker 44: having a molecular weight of about 50.0 kD;
Biomarker 45: having a molecular weight of about 50.1 kD;
Biomarker 46: having a molecular weight of about 51.1 kD;
Biomarker 47: having a molecular weight of about 51.3 kD; and
Biomarker 48: having a molecular weight of about 67.0 kD.

As discussed above, Biomarkers 1 through 48 also may be characterized based on affinity for an adsorbent, particularly binding to an immobilized chelate (IMAC)-Cu substrate surface under the conditions specified under ProteinChip Analysis of the General Comments of the Examples, which follow.

II. TEST SAMPLES

A) SUBJECT TYPES

Samples are collected from subjects, e.g., patients who want to establish kidney transplant rejection status. Other patients include patoients who have kidney transplant rejection and the test is being used to determine the effectiveness of immunosuppressive therapy or treatment they are receiving

B) TYPES OF SAMPLE AND PREPARATION OF THE SAMPLE

The Biomarkers can be measured in different types of biological samples. The sample is preferably a biological fluid sample. Examples of a biological fluid sample useful in this invention include blood, blood serum, plasma, vaginal secretions, urine, tears, saliva, *etc.* Because all of the Biomarkers are found in urine, urine is a preferred sample source for embodiments of the invention.

If desired, the sample can be prepared to enhance detectability of the Biomarkers. For example, to increase the detectability of Biomarkers, a urine sample from the subject can be preferably fractionated by, *e.g.*, Cibacron blue agarose chromatography and single stranded DNA affinity chromatography, anion exchange chromatography, affinity chromatography (e.g., with antibodies) and the like. The method of fractionation depends on the type of detection method used. Any method that enriches for the protein of interest can be used. Sample preparations, such as pre-

fractionation protocols, are optional and may not be necessary to enhance detectability of Biomarkers depending on the methods of detection used. For example, sample preparation may be unnecessary if antibodies that specifically bind Biomarkers are used to detect the presence of Biomarkers in a sample.

Typically, sample preparation involves fractionation of the sample and collection of fractions determined to contain the Biomarkers. Methods of pre-fractionation include, for example, size exclusion chromatography, ion exchange chromatography, heparin chromatography, affinity chromatography, sequential extraction, gel electrophoresis and liquid chromatography. The analytes also may be modified prior to detection. These methods are useful to simplify the sample for further analysis. For example, it can be useful to remove high abundance proteins, such as albumin, from blood before analysis. Examples of methods of fractionation are described in PCT/US03/00531 (incorporated herein in its entirety).

Preferably, the sample is pre-fractionated by anion exchange chromatography. Anion exchange chromatography allows pre-fractionation of the proteins in a sample roughly according to their charge characteristics. For example, a Q anion-exchange resin can be used (*e.g.*, Q HyperD F, Biosepra), and a sample can be sequentially eluted with eluants having different pH's. Anion exchange chromatography allows separation of biomolecules in a sample that are more negatively charged from other types of biomolecules. Proteins that are eluted with an eluant having a high pH is likely to be weakly negatively charged, and a fraction that is eluted with an eluant having a low pH is likely to be strongly negatively charged. Thus, in addition to reducing complexity of a sample, anion exchange chromatography separates proteins according to their binding characteristics.

In preferred embodiments, the urine samples are fractionated via anion exchange chromatography. Signal suppression of lower abundance proteins by high abundance proteins presents a significant challenge to SELDI mass spectrometry. Fractionation of a sample reduces the complexity of the constituents of each fraction. This method can also be used to attempt to isolate high abundance proteins into a fraction, and thereby reduce its signal suppression effect on lower abundance proteins. Anion exchange fractionation separates proteins by their isoelectric point (pI).

Proteins are comprised of amino acids, which are ambivalent-their charge changes based on the pH of the environment to which they are exposed. A protein's pI is the pH at which the protein has no net charge. A protein assumes a neutral charge when the pH of the environment is equivalent to pI of the protein. When the pH rises above the pI of the protein, the protein assumes a net negative charge. Similarly, when the pH of the environment falls below the pI of the protein, the protein has a net positive charge. The urine samples were fractionated according to the protocol set forth in the Examples below to obtain the Biomarkers described herein.

Biomolecules in a sample can also be separated by high-resolution electrophoresis, *e.g.*, one or two-dimensional gel electrophoresis. A fraction containing a Biomarker can be isolated and further analyzed by gas phase ion spectrometry. Preferably, two-dimensional gel electrophoresis is used to generate two-dimensional array of spots of biomolecules, including one or more Biomarkers. *See, e.g.*, Jungblut and Thiede, *Mass Spectr. Rev.* 16:145-162 (1997).

The two-dimensional gel electrophoresis can be performed using methods known in the art. *See, e.g.*, Deutscher ed., *Methods In Enzymology* vol. 182. Typically, biomolecules in a sample are separated by, *e.g.*, isoelectric focusing, during which biomolecules in a sample are separated in a pH gradient until they reach a spot where their net charge is zero (*i.e.*, isoelectric point). This first separation step results in one-dimensional array of biomolecules. The biomolecules in one-dimensional array is further separated using a technique generally distinct from that used in the first separation step. For example, in the second dimension, biomolecules separated by isoelectric focusing are further separated using a polyacrylamide gel, such as polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). SDS-PAGE gel allows further separation based on molecular mass of biomolecules. Typically, two-dimensional gel electrophoresis can separate chemically different biomolecules in the molecular mass range from 1000-200,000 Da within complex mixtures. The pI range of these gels is about 3-10 (wide range gels).

Biomolecules in the two-dimensional array can be detected using any suitable methods known in the art. For example, biomolecules in a gel can be labeled or stained (*e.g.*, Coomassie Blue or silver staining). If gel electrophoresis generates

spots that correspond to the molecular weight of one or more Biomarkers of the invention, the spot can be further analyzed by gas phase ion spectrometry. For example, spots can be excised from the gel and analyzed by gas phase ion spectrometry. Alternatively, the gel containing biomolecules can be transferred to an inert membrane by applying an electric field. Then a spot on the membrane that approximately corresponds to the molecular weight of a Biomarker can be analyzed by gas phase ion spectrometry. In gas phase ion spectrometry, the spots can be analyzed using any suitable techniques, such as MALDI or SELDI (*e.g.*, using ProteinChip® array) as described herein.

Prior to gas phase ion spectrometry analysis, it may be desirable to cleave biomolecules in the spot into smaller fragments using cleaving reagents, such as proteases (*e.g.*, trypsin). The digestion of biomolecules into small fragments provides a mass fingerprint of the biomolecules in the spot, which can be used to determine the identity of Biomarkers if desired.

High performance liquid chromatography (HPLC) can also be used to separate a mixture of biomolecules in a sample based on their different physical properties, such as polarity, charge and size. HPLC instruments typically consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Biomolecules in a sample are separated by injecting an aliquot of the sample onto the column. Different biomolecules in the mixture pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase. A fraction that corresponds to the molecular weight and/or physical properties of one or more Biomarkers can be collected. The fraction can then be analyzed by gas phase ion spectrometry to detect Biomarkers. For example, the spots can be analyzed using either MALDI or SELDI (*e.g.*, using ProteinChip® array) as described herein.

Optionally, a Biomarker can be modified before analysis to improve its resolution or to determine its identity. For example, the Biomarkers may be subject to proteolytic digestion before analysis. Any protease can be used. Proteases, such as trypsin, that are likely to cleave the Biomarkers into a discrete number of fragments

are particularly useful. The fragments that result from digestion function as a fingerprint for the Biomarkers, thereby enabling their detection indirectly. This is particularly useful where there are Biomarkers with similar molecular masses that might be confused for the Biomarker in question. Also, proteolytic fragmentation is useful for high molecular weight Biomarkers because smaller Biomarkers are more easily resolved by mass spectrometry. In another example, biomolecules can be modified to improve detection resolution. For instance, neuraminidase can be used to remove terminal sialic acid residues from glycoproteins to improve binding to an anionic adsorbent (*e.g.*, cationic exchange ProteinChip® arrays) and to improve detection resolution. In another example, the Biomarkers can be modified by the attachment of a tag of particular molecular weight that specifically bind to molecular Biomarkers, further distinguishing them. Optionally, after detecting such modified Biomarkers, the identity of the Biomarkers can be further determined by matching the physical and chemical characteristics of the modified Biomarkers in a protein database (*e.g.*, SwissProt).

III. CAPTURE OF BIOMARKERS

Biomarkers are preferably captured with capture reagents immobilized to a solid support, such as any biochip described herein, a multiwell microtiter plate or a resin. In particular, the Biomarkers of this invention are preferably captured on SELDI protein biochips. Capture can be on a chromatographic surface or a biospecific surface. Any of the SELDI protein biochips comprising reactive surfaces can be used to capture and detect the Biomarkers of this invention. However, the Biomarkers of this invention bind well to immobilized metal chelates. The IMAC-3 and IMAC 30 biochips, which nitriloacetic acid functionalities that adsorb transition metal ions, such as Cu^{++} and Ni^{++}, by chelation, are the preferred SELDI biochips for capturing the Biomarkers of this invention. Any of the SELDI protein biochips comprising reactive surfaces can be used to capture and detect the Biomarkers of this invention. These biochips can be derivatized with the antibodies that specifically capture the Biomarkers, or they can be derivatized with capture reagents, such as protein A or protein G that bind immunoglobulins. Then the Biomarkers can be captured in solution using specific antibodies and the captured Biomarkers isolated on chip through the capture reagent.

In general, a sample containing the Biomarkers, such as serum, is placed on the active surface of a biochip for a sufficient time to allow binding. Then, unbound molecules are washed from the surface using a suitable eluant, such as phosphate buffered saline. In general, the more stringent the eluant, the more tightly the proteins must be bound to be retained after the wash. The retained protein Biomarkers now can be detected by appropriate means.

IV. DETECTION AND MEASUREMENT OF BIOMARKERS

Once captured on a substrate, e.g., biochip or antibody, any suitable method can be used to measure a Biomarker or Biomarkers in a sample. For example, Biomarkers can be detected and/or measured by a variety of detection methods including for example, gas phase ion spectrometry methods, optical methods, electrochemical methods, atomic force microscopy and radio frequency methods. Using these methods, one or more Biomarkers can be detected.

A) SELDI

One preferred method of detection and/or measurement of the Biomarkers uses mass spectrometry and, in particular, "Surface-enhanced laser desorption/ionization" or "SELDI". SELDI refers to a method of desorption/ionization gas phase ion spectrometry (e.g., mass spectrometry) in which the analyte is captured on the surface of a SELDI probe that engages the probe interface. In "SELDI MS," the gas phase ion spectrometer is a mass spectrometer. SELDI technology is described in more detail above.

B) IMMUNOASSAY

In another embodiment, an immunoassay can be used to detect and analyze Biomarkers in a sample. This method comprises: (a) providing an antibody that specifically binds to a Biomarker; (b) contacting a sample with the antibody; and (c) detecting the presence of a complex of the antibody bound to the Biomarker in the sample.

An immunoassay is an assay that uses an antibody to specifically bind an antigen (*e.g.*, a Biomarker). The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the

antigen. The phrase "specifically (or selectively) binds" to an antibody or "specifically (or selectively) immunoreactive with," when referring to a protein or peptide, refers to a binding reaction that is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and do not substantially bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, polyclonal antibodies raised to a Biomarker from specific species such as rat, mouse, or human can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with that Biomarker and not with other proteins, except for polymorphic variants and alleles of the Biomarker. This selection may be achieved by subtracting out antibodies that cross-react with the Biomarker molecules from other species.

Using the purified Biomarkers or their nucleic acid sequences, antibodies that specifically bind to a Biomarker can be prepared using any suitable methods known in the art. *See, e.g.*, Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *Antibodies: A Laboratory Manual* (1988); Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986); and Kohler & Milstein, *Nature* 256:495-497 (1975). Such techniques include, but are not limited to, antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors, as well as preparation of polyclonal and monoclonal antibodies by immunizing rabbits or mice (*see, e.g.*, Huse *et al.*, *Science* 246:1275-1281 (1989); Ward *et al.*, *Nature* 341:544-546 (1989)). Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 to 100 times background.

Generally, a sample obtained from a subject can be contacted with the antibody that specifically binds the Biomarker. Optionally, the antibody can be fixed to a solid support to facilitate washing and subsequent isolation of the complex, prior to contacting the antibody with a sample. Examples of solid supports include glass or plastic in the form of, *e.g.*, a microtiter plate, a stick, a bead, or a microbead. Antibodies can also be attached to a probe substrate or ProteinChip® array described above. The sample is preferably a biological fluid sample taken from a subject.

Examples of biological fluid samples include blood, serum, plasma, nipple aspirate, urine, tears, saliva *etc.* In a preferred embodiment, the biological fluid comprises blood serum. The sample can be diluted with a suitable eluant before contacting the sample to the antibody.

After incubating the sample with antibodies, the mixture is washed and the antibody-Biomarker complex formed can be detected. This can be accomplished by incubating the washed mixture with a detection reagent. This detection reagent may be, *e.g.*, a second antibody which is labeled with a detectable label. Exemplary detectable labels include magnetic beads (*e.g.*, DYNABEADS™), fluorescent dyes, radiolabels, enzymes (*e.g.*, horse radish peroxide, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads. Alternatively, the Biomarker in the sample can be detected using an indirect assay, wherein, for example, a second, labeled antibody is used to detect bound Biomarker-specific antibody, and/or in a competition or inhibition assay wherein, for example, a monoclonal antibody which binds to a distinct epitope of the Biomarker is incubated simultaneously with the mixture.

Methods for measuring the amount of, or presence of, antibody-Biomarker complex include, for example, detection of fluorescence, luminescence, chemiluminescence, absorbance, reflectance, transmittance, birefringence or refractive index (e.g., surface plasmon resonance, ellipsometry, a resonant mirror method, a grating coupler waveguide method or interferometry). Optical methods include microscopy (both confocal and non-confocal), imaging methods and non-imaging methods. Electrochemical methods include voltametry and amperometry methods. Radio frequency methods include multipolar resonance spectroscopy. Methods for performing these assays are readily known in the art. Useful assays include, for example, an enzyme immune assay (EIA) such as enzyme-linked immunosorbent assay (ELISA), a radioimmune assay (RIA), a Western blot assay, or a slot blot assay. These methods are also described in, *e.g.*, *Methods in Cell Biology: Antibodies in Cell Biology*, volume 37 (Asai, ed. 1993); *Basic and Clinical Immunology* (Stites & Terr, eds., 7th ed. 1991); and Harlow & Lane, *supra*.

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, Biomarker, volume of solution, concentrations and the like. Usually the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

Immunoassays can be used to determine presence or absence of a Biomarker in a sample as well as the quantity of a Biomarker in a sample. The amount of an antibody-Biomarker complex can be determined by comparing to a standard. A standard can be, *e.g.*, a known compound or another protein known to be present in a sample. As noted above, the test amount of Biomarker need not be measured in absolute units, as long as the unit of measurement can be compared to a control.

The methods for detecting these Biomarkers in a sample have many applications. For example, one or more Biomarkers can be measured to aid kidney transplant rejection diagnosis or prognosis. In another example, the methods for detection of the Biomarkers can be used to monitor responses in a subject to immunosuppression treatment. In another example, the methods for detecting Biomarkers can be used to assay for and to identify compounds that modulate expression of these Biomarkers *in vivo* or *in vitro*. In a preferred example, the Biomarkers are used to differentiate between the different stages of rejection progression, thus aiding in determining appropriate treatment.

V. DATA ANALYSIS

When the sample is measured and data is generated, e.g., by mass spectrometry, the data is then analyzed by a computer software program. Generally, the software can comprise code that converts signal from the mass spectrometer into computer readable form. The software also can include code that applies an algorithm to the analysis of the signal to determine whether the signal represents a "peak" in the signal corresponding to a Biomarker of this invention, or other useful Biomarkers. The software also can include code that executes an algorithm that compares signal from a test sample to a typical signal characteristic of "normal" and human cancer and

determines the closeness of fit between the two signals. The software also can include code indicating which the test sample is closest to, thereby providing a probable diagnosis.

In preferred methods of the present invention, multiple Biomarkers are measured. The use of multiple Biomarkers increases the predictive value of the test and provides greater utility in diagnosis, toxicology, patient stratification and patient monitoring. The process called "Pattern recognition" detects the patterns formed by multiple Biomarkers greatly improves the sensitivity and specificity of clinical proteomics for predictive medicine. Subtle variations in data from clinical samples, e.g., obtained using SELDI, indicate that certain patterns of protein expression can predict phenotypes such as the presence or absence of a certain stage of rejection or a positive or adverse response to immunosuppression treatments.

Data generation in mass spectrometry begins with the detection of ions by an ion detector as described above. Ions that strike the detector generate an electric potential that is digitized by a high speed time-array recording device that digitally captures the analog signal. Ciphergen's ProteinChip® system employs an analog-to-digital converter (ADC) to accomplish this. The ADC integrates detector output at regularly spaced time intervals into time-dependent bins. The time intervals typically are one to four nanoseconds long. Furthermore, the time-of-flight spectrum ultimately analyzed typically does not represent the signal from a single pulse of ionizing energy against a sample, but rather the sum of signals from a number of pulses. This reduces noise and increases dynamic range. This time-of-flight data is then subject to data processing. In Ciphergen's ProteinChip® software, data processing typically includes TOF-to-M/Z transformation, baseline subtraction, high frequency noise filtering.

TOF-to-M/Z transformation involves the application of an algorithm that transforms times-of-flight into mass-to-charge ratio (M/Z). In this step, the signals are converted from the time domain to the mass domain. That is, each time-of-flight is converted into mass-to-charge ratio, or M/Z. Calibration can be done internally or externally. In internal calibration, the sample analyzed contains one or more analytes of known M/Z. Signal peaks at times-of-flight representing these massed analytes are assigned the known M/Z. Based on these assigned M/Z ratios, parameters are

calculated for a mathematical function that converts times-of-flight to M/Z. In external calibration, a function that converts times-of-flight to M/Z, such as one created by prior internal calibration, is applied to a time-of-flight spectrum without the use of internal calibrants.

Baseline subtraction improves data quantification by eliminating artificial, reproducible instrument offsets that perturb the spectrum. It involves calculating a spectrum baseline using an algorithm that incorporates parameters such as peak width, and then subtracting the baseline from the mass spectrum.

High frequency noise signals are eliminated by the application of a smoothing function. A typical smoothing function applies a moving average function to each time-dependent bin. In an improved version, the moving average filter is a variable width digital filter in which the bandwidth of the filter varies as a function of, e.g., peak bandwidth, generally becoming broader with increased time-of-flight. See, e.g., WO 00/70648, November 23, 2000 (Gavin et al., "Variable Width Digital Filter for Time-of-flight Mass Spectrometry").

Analysis generally involves the identification of peaks in the spectrum that represent signal from an analyte. Peak selection can, of course, be done by eye. However, software is available as part of Ciphergen's ProteinChip® software that can automate the detection of peaks. In general, this software functions by identifying signals having a signal-to-noise ratio above a selected threshold and labeling the mass of the peak at the centroid of the peak signal. In one useful application many spectra are compared to identify identical peaks present in some selected percentage of the mass spectra. One version of this software clusters all peaks appearing in the various spectra within a defined mass range, and assigns a mass (M/Z) to all the peaks that are near the mid-point of the mass (M/Z) cluster.

Peak data from one or more spectra can be subject to further analysis by, for example, creating a spreadsheet in which each row represents a particular mass spectrum, each column represents a peak in the spectra defined by mass, and each cell includes the intensity of the peak in that particular spectrum. Various statistical or pattern recognition approaches can applied to the data.

In one example, Ciphergen's BioBiomarker Patterns™ Software is used to detect a pattern in the spectra that are generated. The data is classified using a pattern recognition process that uses a classification model. In general, the spectra will represent samples from at least two different groups for which a classification algorithm is sought. For example, the groups can be pathological v. non-pathological (e.g., rejection v. non-rejection), drug responder v. drug non-responder, toxic response v. non-toxic response, progressor to disease state v. non-progressor to disease state, phenotypic condition present v. phenotypic condition absent.

The spectra that are generated in embodiments of the invention can be classified using a pattern recognition process that uses a classification model. In some embodiments, data derived from the spectra (*e.g.*, mass spectra or time-of-flight spectra) that are generated using samples such as "known samples" can then be used to "train" a classification model. A "known sample" is a sample that is pre-classified (*e.g.*, rejection or non-rejection). Data derived from the spectra (e.g., mass spectra or time-of-flight spectra) that are generated using samples such as "known samples" can then be used to "train" a classification model. A "known sample" is a sample that is pre-classified. The data that are derived from the spectra and are used to form the classification model can be referred to as a "training data set". Once trained, the classification model can recognize patterns in data derived from spectra generated using unknown samples. The classification model can then be used to classify the unknown samples into classes. This can be useful, for example, in predicting whether or not a particular biological sample is associated with a certain biological condition (e.g., rejection v. non-rejection).

The training data set that is used to form the classification model may comprise raw data or pre-processed data. In some embodiments, raw data can be obtained directly from time-of-flight spectra or mass spectra, and then may be optionally "pre-processed" in any suitable manner. For example, signals above a predetermined signal-to-noise ratio can be selected so that a subset of peaks in a spectrum is selected, rather than selecting all peaks in a spectrum. In another example, a predetermined number of peak "clusters" at a common value (*e.g.*, a particular time-of-flight value or mass-to-charge ratio value) can be used to select peaks. Illustratively, if a peak at a given mass-to-charge ratio is in less than 50% of

the mass spectra in a group of mass spectra, then the peak at that mass-to-charge ratio can be omitted from the training data set. Pre-processing steps such as these can be used to reduce the amount of data that is used to train the classification model.

Classification models can be formed using any suitable statistical classification (or "learning") method that attempts to segregate bodies of data into classes based on objective parameters present in the data. Classification methods may be either supervised or unsupervised. Examples of supervised and unsupervised classification processes are described in Jain, "Statistical Pattern Recognition: A Review", IEEE Transactions on Pattern Analysis and Machine Intelligence, Vol. 22, No. 1, January 2000, which is herein incorporated by reference in its entirety.

In supervised classification, training data containing examples of known categories are presented to a learning mechanism, which learns one more sets of relationships that define each of the known classes. New data may then be applied to the learning mechanism, which then classifies the new data using the learned relationships. Examples of supervised classification processes include linear regression processes (e.g., multiple linear regression (MLR), partial least squares (PLS) regression and principal components regression (PCR)), binary decision trees (e.g., recursive partitioning processes such as CART - classification and regression trees), artificial neural networks such as backpropagation networks, discriminant analyses (e.g., Bayesian classifier or Fischer analysis), logistic classifiers, and support vector classifiers (support vector machines).

A preferred supervised classification method is a recursive partitioning process. Recursive partitioning processes use recursive partitioning trees to classify spectra derived from unknown samples. Further details about recursive partitioning processes are provided in U.S. 2002 0138208 A1 (Paulse et al., "Method for analyzing mass spectra," September 26, 2002.

In other embodiments, the classification models that are created can be formed using unsupervised learning methods. Unsupervised classification attempts to learn classifications based on similarities in the training data set, without pre classifying the spectra from which the training data set was derived. Unsupervised learning methods

include cluster analyses. A cluster analysis attempts to divide the data into "clusters" or groups that ideally should have members that are very similar to each other, and very dissimilar to members of other clusters. Similarity is then measured using some distance metric, which measures the distance between data items, and clusters together data items that are closer to each other. Clustering techniques include the MacQueen's K-means algorithm and the Kohonen's Self-Organizing Map algorithm.

Learning algorithms asserted for use in classifying biological information are described in, for example, WO 01/31580 (Barnhill et al., "Methods and devices for identifying patterns in biological systems and methods of use thereof," May 3, 2001); U.S. 2002/0193950 A1 (Gavin et al., "Method or analyzing mass spectra," December 19, 2002); U.S. 2003/0004402 A1 (Hitt et al., "Process for discriminating between biological states based on hidden patterns from biological data," January 2, 2003); and U.S. 2003/ 0055615 A1 (Zhang and Zhang, "Systems and methods for processing biological expression data" March 20, 2003).

Generally, the data generated from Section IV above is inputted into a diagnostic algorithm (i.e., classification algorithm as described above). The classification algorithm is then generated based on the learning algorithm. The process involves developing an algorithm that can generate the classification algorithm. The methods of the present invention generate a more accurate classification algorithm by accessing a number of kidney transplant rejection and normal samples of a sufficient number based on statistical sample calculations. The samples are used as a training set of data on learning algorithm.

The generation of the classification, i.e., diagnostic, algorithm is dependent upon the assay protocol used to analyze samples and generate the data obtained in Section IV above. It is imperative that the protocol for the detection and/or measurement of the Biomarkers (e.g., in step IV) must be the same as that used to obtain the data used for developing the classification algorithm. The assay conditions, which must be maintained throughout the training and classification systems include chip type and mass spectrometer parameters, as well as general protocols for sample preparation and testing. If the protocol for the detection and/or measurement of the Biomarkers (step IV) is changed, the learning algorithm and classification algorithm must also change. Similarly, if the learning algorithm and classification algorithm

change, then the protocol for the detection and/or measurement of Biomarkers (step IV) must also change to be consistent with that used to generate classification algorithm. Development of a new classification model would require accessing a sufficient number of kidney transplant rejection and non-rejection samples, developing a new training set of data based on a new detection protocol, generating a new classification algorithm using the data and finally, verifying the classification algorithm with a multi-site study.

The classification models can be formed on and used on any suitable digital computer. Suitable digital computers include micro, mini, or large computers using any standard or specialized operating system such as a Unix, Windows™ or Linux™ based operating system. The digital computer that is used may be physically separate from the mass spectrometer that is used to create the spectra of interest, or it may be coupled to the mass spectrometer. If it is separate from the mass spectrometer, the data must be inputted into the computer by some other means, whether manually or automated.

The training data set and the classification models according to embodiments of the invention can be embodied by computer code that is executed or used by a digital computer. The computer code can be stored on any suitable computer readable media including optical or magnetic disks, sticks, tapes, etc., and can be written in any suitable computer programming language including C, C++, visual basic, etc.

VI. EXAMPLES OF PREFERRED EMBODIMENTS.

In a preferred embodiment, a urine sample is collected from a patient and then fractionated using an anion exchange resin as described above. The Biomarkers in the sample are captured using an IMAC copper ProteinChip array. The Biomarkers are then detected using SELDI. The results are then entered into a computer system, which contains an algorithm that is designed using the same parameters that were used in the learning algorithm and classification algorithm to originally determine the Biomarkers. The algorithm produces a diagnosis based upon the data received relating to each Biomarker.

The diagnosis is determined by examining the data produced from the SELDI tests with the classification algorithm that is developed using the Biomarkers. The classification algorithm depends on the particulars of the test protocol used to detect the Biomarkers. These particulars include, for example, sample preparation, chip type and mass spectrometer parameters. If the test parameters change, the algorithm must change. Similarly, if the algorithm changes, the test protocol must change.

In another embodiment, the sample is collected from the patient. The Biomarkers are captured using an antibody ProteinChip array as described above. The Biomarkers are detected using a biospecific SELDI test system The results are then entered into a computer system, which contains an algorithm that is designed using the same parameters that were used in the learning algorithm and classification algorithm to originally determine the Biomarkers. The algorithm produces a diagnosis based upon the data received relating to each Biomarker.

In yet other preferred embodiments, the Biomarkers are captured and tested using non-SELDI formats. In one example, the sample is collected from the patient. The Biomarkers are captured on a substrate using other known means, e.g., antibodies to the Biomarkers. The Biomarkers are detected using methods known in the art, e.g., optical methods and refractive index. Examples of optical methods include detection of fluorescence, e.g., ELISA. Examples of refractive index include surface plasmon resonance. The results for the Biomarkers are then subjected to an algorithm, which may or may not require artificial intelligence. The algorithm produces a diagnosis based upon the data received relating to each Biomarker.

In any of the above methods, the data from the sample may be fed directly from the detection means into a computer containing the diagnostic algorithm. Alternatively, the data obtained can be fed manually, or via an automated means, into a separate computer that contains the diagnostic algorithm.

VII. DIAGNOSIS OF SUBJECT AND DETERMINATION OF KIDNEY TRANSPLANT REJECTION STATUS

Any Biomarker, individually, is useful in aiding in the determination of kidney transplant rejection status. First, the selected Biomarker is measured in a subject sample using the methods described herein, e.g., capture on a SELDI biochip

followed by detection by mass spectrometry. Then, the measurement is compared with a diagnostic amount or control that distinguishes kidney transplant rejection status from a non-rejection status. The diagnostic amount will reflect the information herein that a particular Biomarker is up-regulated or down-regulated in a kidney transplant rejection status compared with a non-rejection status. As is well understood in the art, the particular diagnostic amount used can be adjusted to increase sensitivity or specificity of the diagnostic assay depending on the preference of the diagnostician. The test amount as compared with the diagnostic amount thus indicates kidney transplant rejection status.

While individual Biomarkers are useful diagnostic Biomarkers, it has been found that a combination of Biomarkers provides greater predictive value than single Biomarkers alone. Specifically, the detection of a plurality of Biomarkers in a sample increases the percentage of true positive and true negative diagnoses and would decrease the percentage of false positive or false negative diagnoses. Thus, preferred methods of the present invention comprise the measurement of more than one Biomarker. For example, the methods of the present invention have an AUC from ROC analysis greater than 0.50, more preferred methods have an AUC greater than 0.60, more preferred methods have an AUC greater than 0.70. Especially preferred methods have an AUC greater than 0.70 and most preferred methods have an AUC greater than 0.80.

Furthermore, using a method that measures the combination of the 48 preferred Biomarkers of the present invention significantly improves the diagnostic performance, providing a test that has an AUC greater than 0.50, more preferred tests have an AUC greater than 0.60, more preferred tests have an AUC greater than 0.70.

In order to use the Biomarkers in combination, a logistical regression algorithm is useful. The UMSA algorithm is particularly useful to generate a diagnostic algorithm from test data. This algorithm is disclosed in Z. Zhang et al., Applying classification separability analysis to microarray data. In: Lin SM, Johnson KF, eds. Methods of Microarray data analysis: papers from CAMDA '00. Boston: Kluwer Academic Publishers, 2001:125-136; and Z. Zhang et al., Fishing Expedition – a Supervised Approach to Extract Patterns from a Compendium of Expression

Profiles. In Lin SM, Johnson, KF, eds. Microarray Data Analysis II: Papers from CAMDA '01. Boston: Kluwer Academic Publishers, 2002.

The learning algorithm will generate a multivariate classification (diagnostic) algorithm tuned to the particular specificity and sensitivity desired by the operator. The classification algorithm can then be used to determine kidney transplant rejection status. The method also involves measuring the selected Biomarkers in a subject sample (e.g., Biomarkers 1 through 48). These measurements are submitted to the classification algorithm. The classification algorithm generates an indicator score that indicates kidney transplant rejection status.

In some embodiments, the mere presence or absence of a Biomarker, without quantifying the amount of Biomarker, is useful and can be correlated with a probable diagnosis of kidney transplant rejection. For example, Biomarker 15 can be more frequently detected in human kidney transplant rejection patients than in non-rejection patients. Equally, for example, Biomarkers 29 and 30, can be less frequently detected in human kidney transplant rejection patients than in non-rejection patients. Thus, a detected presence or absence, respectively, of these Biomarkers in a subject being tested indicates that the subject has a higher probability of having kidney transplant rejection.

In other embodiments, the measurement of Biomarkers can involve quantifying the Biomarkers to correlate the detection of Biomarkers with a probable diagnosis of kidney transplant rejection. Thus, if the amount of the Biomarkers detected in a subject being tested is different compared to a control amount (i.e., higher or lower than the control, depending on the Biomarker), then the subject being tested has a higher probability of having kidney transplant rejection.

The correlation may take into account the amount of the Biomarker or Biomarkers in the sample compared to a control amount of the Biomarker or Biomarkers (up or down regulation of the Biomarker or Biomarkers) (*e.g.*, in normal subjects in whom human cancer is undetectable). A control can be, *e.g.*, the average or median amount of Biomarker present in comparable samples of normal subjects in whom rejection is undetectable. The control amount is measured under the same or

substantially similar experimental conditions as in measuring the test amount. The correlation may take into account the presence or absence of the Biomarkers in a test sample and the frequency of detection of the same Biomarkers in a control. The correlation may take into account both of such factors to facilitate determination of kidney transplant rejection status.

In certain embodiments of the methods of qualifying kidney transplant rejection status, the methods further comprise managing subject treatment based on the status. As aforesaid, such management describes the actions of the physician or clinician subsequent to determining kidney transplant rejection status. For example, if the result of the methods of the present invention is inconclusive or there is reason that confirmation of status is necessary, the physician may order more tests. Alternatively, if the status indicates that altered immunosuppression therapy is appropriate, the physician may schedule the patient for a change in therapy. Likewise, if the result is negative, e.g., the status indicates late stage kidney transplant rejection or if the status is otherwise acute, no further action may be warranted. Furthermore, if the results show that treatment has been successful, no further management may be necessary.

The invention also provides for such methods where the Biomarkers (or specific combination of Biomarkers) are measured again after subject management. In these cases, the methods are used to monitor the status of the rejection, e.g., response to immunosuppression treatment. Because of the ease of use of the methods and the lack of invasiveness of the methods, the methods can be repeated after each treatment the patient receives. This allows the physician to follow the effectiveness of the course of treatment. If the results show that the treatment is not effective, the course of treatment can be altered accordingly. This enables the physician to be flexible in the treatment options.

In another example, the methods for detecting Biomarkers can be used to assay for and to identify compounds that modulate expression of these Biomarkers *in vivo* or *in vitro*.

The methods of the present invention have other applications as well. For example, the Biomarkers can be used to screen for compounds that modulate the expression of the Biomarkers *in vitro* or *in vivo*, which compounds in turn may be useful in treating or preventing kidney transplant rejection in patients. In another example, the Biomarkers can be used to monitor the response to treatments for kidney transplant rejection

VIII. KITS

In yet another aspect, the present invention provides kits for qualifying kidney transplant rejection status, wherein the kits can be used to measure the Biomarkers of the present invention. For example, the kits can be used to measure any one or more of the Biomarkers described herein, which Biomarkers are differentially present in samples of kidney transplant rejection patient and non-rejection patients. The kits of the invention have many applications. For example, the kits can be used to differentiate if a subject has kidney transplant rejection or has a negative diagnosis, thus enabling the physician or clinician to diagnose the presence or absence of rejection. The kits can also be used to monitor the patient's response to a course of treatment, enabling the physician to modify the treatment based upon the results of the test. In another example, the kits can be used to identify compounds that modulate expression of one or more of the Biomarkers in *in vitro* or *in vivo* animal models for kidney transplant rejection.

The present invention therefore provides kits comprising (a) a capture reagent that binds a Biomarker selected from Biomarkers 1 through 48, and combinations thereof; and (b) a container comprising at least one of the Biomarkers. In preferred kit, the capture reagent binds a plurality of the Biomarkers. In certain preferred embodiments, the kit of further comprises a second capture reagent that binds one of the Biomarkers that the first capture reagent does not bind.

Further kits provided by the invention comprise (a) a first capture reagent that binds at least one Biomarker selected from Biomarkers 1 through 48 and (b) a second capture reagent that binds at least one of the Biomarkers that is not bound by the first capture reagent. Preferably, at least one of the capture reagents is an antibody.

Certain kits further comprise an MS probe to which at least one capture reagent is attached or is attachable.

While the capture reagent can be any type of reagent, preferably the reagent is a SELDI probe. In certain kits of the present invention, the capture reagent comprises an IMAC.

The invention also provides kits comprising (a) a first capture reagent that binds at least one Biomarker selected from Biomarkers 1 through 48 and (b) instructions for using the capture reagent to measure the Biomarker. In certain of these kits, the capture reagent comprises an antibody. Furthermore, some of the aforesaid kits further comprise an MS probe to which the capture reagent is attached or is attachable. In some kits, the capture reagent comprises an IMAC. Each of the Biomarkers identified here binds to the IMAC ProteinChip® array. Therefore, one preferred embodiment of the present invention includes a high-throughput test for early detection of kidney transplant rejection, which analyzes a patient's sample on the IMAC ProteinChip® array for these analytes.

In other embodiments, the kits as described herein comprise at least one capture reagent that binds at least one Biomarker selected from Biomarkers 1 through 48.

Certain kits of the present invention further comprise a wash solution, or eluant, that selectively allows retention of the bound Biomarker to the capture reagent as compared with other Biomarkers after washing. Alternatively, the kit may contain instructions for making a wash solution, wherein the combination of the adsorbent and the wash solution allows detection of the Biomarkers using gas phase ion spectrometry.

Preferably, the kit comprises written instructions for use of the kit for detection of kidney transplant rejection and the instructions provide for contacting a test sample with the capture reagent and detecting one or more Biomarkers retained by the capture reagent. For example, the kit may have standard instructions informing a technician how to wash the capture reagent (e.g., probe) after a sample of urine serum contacts the capture reagent. In another example, the kit may have

instructions for pre-fractionating a sample to reduce complexity of proteins in the sample. In another example, the kit may have instructions for automating the fractionation or other processes.

Such kits can be prepared from the materials described above, and the previous discussion of these materials (*e.g.*, probe substrates, capture reagents, adsorbents, washing solutions, *etc.*) is fully applicable to this section and will not be repeated.

In another embodiment, the kit may comprise a first substrate comprising an adsorbent thereon (*e.g.*, a particle functionalized with an adsorbent) and a second substrate onto which the first substrate can be positioned to form a probe, which is removably insertable into a gas phase ion spectrometer. In other embodiments, the kit may comprise a single substrate, which is in the form of a removably insertable probe with adsorbents on the substrate. In yet another embodiment, the kit may further comprise a pre-fractionation spin column (*e.g.*, Cibacron blue agarose column, anti-HSA agarose column, K-30 size exclusion column, Q-anion exchange spin column, single stranded DNA column, lectin column, *etc.*).

In another embodiment, a kit comprises (a) an antibody that specifically binds to a Biomarker; and (b) a detection reagent. Such kits can be prepared from the materials described above, and the previous discussion regarding the materials (*e.g.*, antibodies, detection reagents, immobilized supports, *etc.*) is fully applicable to this section and will not be repeated. Optionally, the kit may further comprise pre-fractionation spin columns. In some embodiments, the kit may further comprise instructions for suitable operation parameters in the form of a label or a separate insert.

Optionally, the kit may further comprise a standard or control information so that the test sample can be compared with the control information standard to determine if the test amount of a Biomarker detected in a sample is a diagnostic amount consistent with a diagnosis of kidney transplant rejection.

The invention also provides an article manufacture comprising at least one capture reagent bound to at least two Biomarkers selected from Biomarkers 1 through 48. Examples of articles of manufacture of the present invention include, but are not limited to, ProteinChip® Arrays, probes, microtitre plates, beads, test tubes, microtubes, and any other solid phase onto which a capture reagent can be incorporated. In an example of such an article, a ProteinChip® Array for example, will have an adsorbent that will capture Biomarkers 1 through 48. These are a few examples of such articles of manufacture. One of ordinary skill in the art would readily be able to manufacture other such articles in accordance with the teachings described herein.

The present invention also provides a system comprising a plurality of capture reagents each of which has bound to it a different Biomarker selected from Biomarkers 1 through 48. An example of such a system includes, but is not limited to, a set of ProteinChip® Arrays, which comprise adsorbents that bind one or more of the Biomarkers selected from Biomarkers 1 through 48. In this type of system, there may be one ProteinChip® Array for each of the Biomarkers Examples of other systems include those in which the capture reagents are test tubes containing an antibody for each of the Biomarkers, either separately, or in groups. One of ordinary skill in the art would readily be able to manufacture other such articles in accordance with the teachings described herein.

The following example is offered by way of illustration, not by way of limitation. Whilea specific example has been provided, the above description is illustrative and not restrictive. Any one or more of the features of the previously described embodiments can be combined in any manner with one or more features of any other embodiments in the present invention. Furthermore, many variations of the invention will become apparent to those skilled in the art upon review of the specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if

each individual publication or patent document were so individually denoted. By their citation of various references in this document, Applicants do not admit any particular reference is "prior art" to their invention.

EXAMPLE

Materials and Methods

Patient Samples

Thirty-four urine samples were collected from 32 renal transplant patients at various stages posttransplantation. Samples were collected from 17 transplant recipients with acute rejection and 15 patients with no rejection. Two patients had paired samples collected before and during a rejection episode. Samples from patients less than 4 days posttransplant were not accepted for data analysis due to the presence of excessive inflammatory response proteins. All cases of rejection were confirmed by biopsy specimens evaluated by an independent, blinded pathologist. Banff 97 classification criteria were used for diagnosis.

Urine Processing

Specimens were centrifuged for 5 minutes at 1,000*g* to remove sediment. Supernatants were aliquoted and frozen at -80°C.

Surface Enhanced Laser Desorption Ionization (SELDI) Mass Spectrometry

Processed urine samples were analyzed in triplicate using SELDI(Kuwata H, Yip TT, Yip CL, et al. Bactericidal domain of lactoferrin: detection, quantitation, and characterization of lactoferricin in serum by SELDI affinity mass spectrometry. Biochem Biophys Res Commun. 1998; 245: 764.). and ProteinChip Arrays,(Rai AJ, Zhang Z, Rosenzweig J, et al. Proteomic approaches to tumor marker discovery: Identification of biomarkers for ovarian cancer. Arch Pathol Lab Med. 2002; 126: 1518; and Li J, Zhang Z, Rosenzweig J, et al. Proteomics and bioinformatics approaches for identification of serum biomarkers to detect breast cancer. Clin Chem. 2002; 48: 1296.) with immobilized metal affinity (IMAC-3) and hydrophobic (H4) surface chemistry. IMAC-3 chips were pretreated with 100 mmol/L $CuSO_4$ and phosphate-buffered saline (PBS) at pH 7.4. H4 chips were pretreated with 50%

acetonitrile. Three microliters of urine were added to each chip spot in duplicate. Chips were incubated at 37°C between applications, allowing samples to dry on the chip surface. Specimens were applied to chips in a random pattern to minimize the effects of spot-to-spot variation. Following sample application, IMAC-3 chips were washed with PBS and H4 chips were washed with 20% acetonitrile to remove nonspecific binding components. CHCA (α-cyano-4-hydroxycinnamic acid) or SPA (sinapinic acid) matrix solution (composed of energy-absorbing molecules) was then added to each chip spot in duplicate. Protein chips were analyzed on a PBS-II mass reader (Ciphergen Biosystems, Fremont, CA) with SELDI 3.0 software. Data were collected by averaging 110 laser shots, with laser intensities and detector sensitivities optimized for each combination of chip and matrix type.

Data Analysis

Mass spectra generated by SELDI mass spectrometry analysis were examined visually to select and label peaks (Fig. 50) with potential to distinguish between prerejection and rejection patients. In addition, SELDI software was used to identify and label all peaks in the spectrum data by applying a threshold to signal-to-noise values. Labeled peaks were normalized to the creatinine content of each urine specimen, through division of peak intensity by creatinine concentration in g/dL.(Lemann J Jr, Doumas BT. Proteinuria in health and disease assessed by measuring the urinary protein/creatinine ratio. Clin Chem. 1987; 23: 297; Yamaguchi T, Kadono K. Clinical evaluation of the albumin/creatinine ratio in outpatients with diabetes. Nippon Jinzo Gakkai Shi. 1991; 33: 283; and Tomg S, Rigatto C, Rush DN, et al. The urine protein to creatinine ratio (P/C) as a predictor of 24-hour urine protein excretion in renal transplant patients. Transplantation. 2002; 72: 1453.). Outliers were determined statistically and removed from the triplicate data sets based on the results of T_n tests. In the T_n test, a suspected outlier is compared to the overall mean of the data set by subtracting the result in question from the overall mean and dividing by the standard deviation to obtain a T_n value. If the T_n value is greater than the critical T value (obtained from a table), then the result in question is deemed to be an outlier and not included in the average.

Both visually and computer-labeled peaks were analyzed with ProPeak software (3Z Informatics, Mt. Pleasant, SC) to statistically identify those peaks with the best ability

to distinguish between the patient populations. ProPeak software used UMSA (Unified Maximum Separability Analysis)(Zhang Z, Page G, Zhang H. Applying classification separability analysis to microarray data. In: Lin SM, Johnson KF, eds. Methods of Microarray Data Analysis: Papers from CAMDA '00. Boston: Kluwer Academic Publishers; 2001: 25-26; and Vapnik VN. Statistical Learning Theory. New York: John Wiley & Sons; 1998: 401-440.) to identify a direction in n-dimensional space along which two data sets are optimally separated. Bootstrap selection ranked peaks according to the strength and consistency of their ability to discriminate between the sets. Peak intensities were log normalized for ProPeak analysis.

The diagnostic performance of highly ranked peaks from UMSA analysis was evaluated by receiver operator characteristic (ROC) curve analysis (Fig. 51). The ability of the peaks to distinguish between rejection and nonrejection patients was ranked by the area under the ROC curve (AUC). Peaks with AUCs greater than 0.6 were classified as peaks of interest, the highest ones of which (AUCs > 0.75) are considered candidate biomarkers. Computer-labeled peaks were also subjected to a separate CART (Classification and Regression Tree) analysis,(Breiman L, Friedman JH, Olshen RA, et al. Classification and Regression Trees. Monterey, CA: Wadsworths & Brooks; 1984.) implemented by Ciphergen Biomarker Patterns Software, to identify patterns of biomarkers that distinguish between patient populations.

Results

Visual and UMSA analyses of spectra from renal transplant patients revealed 48 peaks of interest (AUCs > 0.600) that showed ability to distinguish between rejection and nonrejection urine samples. From these peaks of interest, 16 peaks (AUCs > 0.750 and $P < 0.0001$-0.0009) showed promise as candidate biomarkers for transplant rejection. Thirteen of these peaks (3.4, 4.1, 6.5, 6.6, 6.7, 7.0, 7.1, 7.3, 7.5, 7.8, 8.0, 10.8, and 13.4 kd) were present in a majority of rejection urine samples but absent from most nonrejection specimens. Three peaks (9.0, 9.7, and 9.8) were downregulated with onset of transplant rejection.

A separate analysis using the CART algorithm in the Ciphergen Biomarker Pattern Software correctly classified 91% of the 34 specimens in the training set, giving a

sensitivity of 83% and specificity of 100% on the same training data set using two separate biomarker candidates at 10.0 kd and 3.4 kd. This result is significant because it demonstrates the potential improvement obtained by combining rejection biomarker candidates into a marker panel.

5